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Analysis of Genetic Alterations in Uterine Leiomyomas and Leiomyosarcomas by Comparative Genomic Hybridization

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Uterine leiomyomas are the most prevalent tumor type in women of reproductive age and are the most common reason for hysterectomies. Although uterine leiomyomas are considered to be benign, they are a major public health concern for women. In contrast, leiomyosarcomas are rare but highly malignant uterine tumors. They may arise in uteri with preexisting leiomyomas and histologically sometimes resemble leiomyomas, thus causing controversy about whether leiomyosarcomas arise within leiomyomas. In this study, we used comparative genomic hybridization (CGH) to identify genetic alterations unique to each tumor type and alterations that are common between the two tumors. We analyzed 14 cases of uterine leiomyomas and eight cases of uterine leiomyosarcomas. Only two of the 14 leiomyomas exhibited genetic alterations, and those were restricted to gains on chromosomes 14 and 19 and losses on chromosomes 1 and 4. In addition, 68 leiomyomas were examined for loss of heterozygosity on chromosomes 1 and 4, and only three tumors exhibited any losses. In contrast, all eight leiomyosarcomas showed gains and losses of DNA by CGH, and in many cases multiple changes were observed. The most commonly observed genetic aberration, occurring in five tumors, was gains on both arms of chromosome 1, suggesting that this chromosome contains loci involved in the development of leiomyosarcoma. Our results do not provide evidence for the progression from benign leiomyoma to malignant leiomyosarcoma. Moreover, the large number of random chromosomal alterations in the leiomyosarcomas suggests that increased genetic instability plays a role in the formation of these tumors. *Mol. Carcinog. 19:273–279, 1997.*

Key words: leiomyoma; comparative genomic hybridization; uterine tumors; sarcomas; molecular cytogenetics

INTRODUCTION

Leiomyomas are benign tumors of the uterus that arise clonally from smooth muscle cells of the myometrium [1,2]. They pose a major health concern in women of reproductive age, being present in 20–30% of all women over the age of 30 [3,4]. However, twice as many African-American women as white women seem to develop this tumor during their reproductive years [5,6]. Multiple leiomyomas of various sizes are often observed in a single uterus. These tumors may grow to be massive, causing severe pain, excessive bleeding, and infertility [7], and they are one of the most common reasons for hysterectomies in the United States [8]. Leiomyomas appear to be hormonally regulated; they occur only after puberty and atrophy during menopause [9]. Despite the importance of these tumors to women's health, knowledge of their pathobiology is limited.

Leiomyosarcoma, the malignant counterpart of leiomyoma, is rare, making up only 1–3% of all malignant uterine neoplasms. Leiomyosarcomas are aggressive tumors with a 5-yr survival of only 20–30%. Although their etiology is unclear, leiomyosarcomas usually occur during menopause in women over the age of 40 [1,3]. Histologically, leiomyosarcomas often resemble leiomyomas, providing support for the controversial hypothesis that leiomyosarcomas arise from preexisting degenerating leiomyomas [3]. The similarity between these two tumors can cause difficulty in diagnosis, and early-stage leiomyosarcomas are sometimes misdiagnosed as leiomyomas.

To understand the pathogenesis of these uterine tumors, we have begun to examine their molecular aberrations. We used the technique of comparative genomic hybridization (CGH) [10] to investigate ge-

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Abbreviations: CGH, comparative genomic hybridization; LOH, loss of heterozygosity

netic gains and losses in these two tumor types. To complement this approach, we analyzed 68 leiomyomas for loss of heterozygosity by using simple sequence repeat markers on chromosomes 1 and 4 in regions that appeared to have DNA losses by CGH. By comparing the results for leiomyomas and leiomyosarcomas, we hope to identify characteristic genetic changes that distinguish the two tumors.

MATERIALS AND METHODS

Tissue Acquisition and DNA Preparation

Ten primary uterine leiomyomas and eight leiomyosarcomas were obtained as frozen specimens from the Department of Obstetrics and Gynecology at Duke University Medical Center (Durham, NC). DNA was isolated from the tissues by the standard procedures of proteinase K digestion and phenol-chloroform extraction [11]. Normal reference DNA was prepared from the peripheral blood lymphocytes of a cytogenetically normal male. DNA samples were quantitated with a Gene Quant 2 (Pharmacia Biotech, Inc., Piscataway, NJ).

Histology

Tissue samples were routinely fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at approximately 6–8 μm. The sections were stained with hematoxylin and eosin and evaluated by using conventional light microscopy.

Metaphase Preparation

Reference metaphase spreads were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes from a healthy male donor (46,XY) by standard procedures. Metaphase slides and spreads for CGH were selected based on the criteria defined by du Manoir et al. [12].

CGH Hybridization

The hybridization was performed as described previously by du Manoir et al. [13]. Briefly, 200 ng of genomic DNA from each test sample was labeled with biotin-16-dUTP by using a standard nick-translation reaction. The same amount of DNA from a normal male was labeled with digoxigenin-11-dUTP and used as the reference DNA. The labeled DNAs were mixed together and hybridized to a normal metaphase spread in the presence of 30 μg of Cot-1 DNA (GIBCO BRL, Gaithersburg, MD) and 10 μg of salmon sperm DNA. The samples were hybridized at 37°C for 3–4 d. Post-hybridization washes and probe detection were performed as described previously by Ried et al. [14].

Digital Image Analysis

Gray-level images were acquired with a Leica DMRBE epifluorescence microscope equipped with a cooled charge-coupled device camera (Photometrics, Tucson, AZ). Gray-level images were taken separately with fluorochrome-specific filters (Chroma Technologies, Brattleboro, VT) for fluorescein (fluorescein isothiocyanate) bound to the biotin-labeled tumor DNA and rhodamine (tetramethylrhodamine B isothiocyanate) bound to the digoxigenin-labeled reference DNA. Chromosomes were identified by 4´,6-diamidino-2-phenylindole (DAPI) banding. Fluorescence ratio images were analyzed as described previously, and the ratio profiles of individual reference chromosomes were determined with a CGH image analysis computer program by du Manoir et al. [13] run on a Macintosh Quadra 950. For each tumor sample the average fluorescein:rhodamine ratio image was calculated from six to 10 metaphases.

Loss of Heterozygosity Analysis

DNA samples from 68 leiomyomas from 15 different individuals were tested for loss of heterozygosity (LOH) on chromosomes 1 and 4. Tumors and corresponding normal tissues were excised after hysterectomy and immediately frozen in liquid nitrogen. DNA was isolated by standard proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation.

The following microsatellite markers were obtained from Research Genetics (Huntsville, AL) to detect LOH in chromosomes 1 and 4: *D1S2134, D1S549, D1S1665, D1S1679, D1S1612, D1S518, D1S1609, D1S534, D1S1588, D1S1622, D1S2130, D4S2623, D4S2366, D4S1627, D4S2367,* and *D4S1625.*

One amplification primer for each marker was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and used for polymerase chain reaction amplification with 25 ng of DNA, 0.2 mM each dNTP, 0.5 μM unlabeled reverse primer, polymerase chain reaction buffer (containing 100 mM Tris-HCL, 500 mM KCL, 15 mM $MgCl_2$, and 0.01% gelatin), and 60 U of Amplitaq Gold Polymerase (Perkin Elmer, Norwalk, CT).

The polymerase chain reactions were conducted by using a touchdown cycling procedure of denaturing at 95°C for 5–10 min followed by 95°C for 20 s and annealing for 20 s at the following temperatures: five cycles at 65°C, five cycles at 60°C, five cycles at 55°C, and 25 cycles at 50°C. Extension steps were performed at 72°C for 30 s. The amplified products were diluted 1:1 with denaturing loading buffer, and samples were denatured at 95°C for 5 min.

Six microliters of each sample was loaded onto 7% polyacrylamide denaturing gels containing 8.3 M urea and 32% formamide and electrophoresed for 3–3.5 h at 90 W. The gels were fixed in 10% methanol and 10% acetic acid, covered with mylar, dried, and exposed to a phosphor-image screen overnight. Alleles were quantitated with a Molecular Dynamics Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

The tumors to be analyzed by CGH were first examined histologically. All of the leiomyomas had

typical histomorphic features consisting of bundles of well-differentiated, spindle-shaped smooth muscle cells with elongated uniform nuclei. Mitotic figures were rare in the uterine leiomyomas. The leiomyosarcomas consisted of anaplastic tumor cells that were disorganized and pleomorphic. The nuclei of the leiomyosarcoma cells were large and irregularly shaped. Mitotic figures were commonly observed within these tumors (data not shown). The grades and stages of the leiomyosarcomas are listed in Table 1.

We used CGH to compare DNA sequence copy number differences in 10 uterine leiomyomas and eight uterine leiomyosarcomas. A summary of the changes identified in both tumor types is shown in Figure 1. Two of the leiomyomas showed genetic changes: sample 3, which had a gain on 19q, and sample 4, which had losses on 1p and 4p (Figures 1 and 2A) and a gain on 14q. Leiomyoma samples 3 and 4 were from patients who had multiple uterine leiomyomas. Two additional tumors from each patient were tested to determine if similar changes existed. No DNA copy number changes were detected by CGH in these four additional leiomyomas.

To further examine the frequency of chromosome 1 and 4 deletions in the uterine leiomyomas, LOH analysis using polymorphic simple-sequence-repeat markers was performed on 68 typical leiomyomas from 15 individuals. The 14 leiomyomas analyzed by CGH were included in these 68 tumors. Three of the tumors had losses on chromosome 1, two on 1q (these two tumors were not analyzed by CGH) and one on 1p. Only one of the 68 tumors showed a 4p loss (Figure 3B), and this was the same tumor in which loss was detected on 1p by both CGH and LOH (Figure 3A).

Genetic changes were revealed by CGH in all eight uterine leiomyosarcomas. Six of the eight malignant tumors (75%) exhibited losses or gains on chromosome 1 (Figures 1 and 2B). Four tumors showed gains on both 1p and 1q; three of the tumors had a common region of overlap of 1q12-q31. Two of the four tumors exhibited gains of the entire 1p arm. Two tumors showed a loss on 1p with a common region of overlap of 1p34.2-pter. An interesting finding for samples 3 and 5 was the CGH correlate of karyotypic isochromosome formation: in sample 5 there was a loss of 6p and a gain of 6q and in sample 3 a loss of 9p and gain of 9q. High levels of amplification on chromosome 6 (sample 1, 6q22-qter) and chromosome 13 (sample 5, 13q31-qter) were demonstrated in two tumors. Other changes detected in the leiomyosarcomas are summarized in Table 1.

DISCUSSION

To our knowledge, this is the first study in which a large number of uterine leiomyomas and leiomyosarcomas have been examined by CGH for chromosomal alterations. This analysis revealed that only 20% of leiomyomas contained DNA sequence copy number changes by this method, whereas all of the leiomyosarcomas exhibited gains and losses of genetic material. In contrast, studies using cytogenetic analysis have shown that many uterine leiomyomas appear cytogenetically abnormal [2]. Those studies detected several nonrandom chromosomal alterations in approximately 25–50% of samples, although much karyotypic heterogeneity existed between tumors [15]. The main cytogenetic alterations observed for leiomyomas have been reciprocal translocations involving chromosomes 12 and 14, a rearrangement at 12q13-q15, deletions of chromosomes 7 and 13, trisomy 12, rearrangements involving the long arm of chromosome 6, and deletions and rearrangements on chromosome 1 [15–18]. Standard

Table 1. Gains and Losses Detected by CGH in Eight Primary Leiomyosarcomas and Two Leiomyomas*

Case no.	Tumor type	Stage/grade	Regional copy number gains	Regional losses
1	Leiomyosarcoma	I	1p11-pter, 1q21-q31, 2p22-pter, 3p21-pter, 3q11.2-qter, 6p11.1-p22, 6q12-q16, ++6q22-qter, 7p11.1-qter, 8q11.2-qter, 11p11.1-pter, 12p11.1-pter, 15q11.1-q23, 16p11.1-pter, 17q22-q25, **18, 19, 21**	None
2	Leiomyosarcoma	III/A	11q22	None
3	Leiomyosarcoma	IV	9q13-qter, 14q11.1-q22, 16p11.1-p13.2, 18p11.1-p11.2, 18q11.1-q22, **21**	9p12-pter
4	Leiomyosarcoma	IV	1p21-pter, 4q14-q22, 8p11.1-pter	6p21.1-pter, 11p15-pter, 13q11-qter, **10**
5	Leiomyosarcoma	I/C	1q21-q32, 3p21-pter, 6q14-qter, 7q31-q35, ++13q31-qter, 19p12-pter	2q23-qter, 5q21-q27, 6p11.1-pter, 9p11-pter, **11**
6	Leiomyosarcoma	IV/B	1p11-pter, 20p11.1-pter	None
7	Leiomyosarcoma	III/C	1q21-q41, **2**	None
8	Leiomyosarcoma	Unknown	13q22-q31, 20q11.1-qter	1p34.2-pter, **17**
3L	Leiomyoma	Unknown	19p12-pter, 9q12-q13.1	None
4L	Leiomyoma	Unknown	14q11.1-q21, 14q24-qter	1p31-pter, 4p11-pter

*++ indicates amplification (ratio value > 1.25). Boldface type indicates whole chromosome gains or losses.

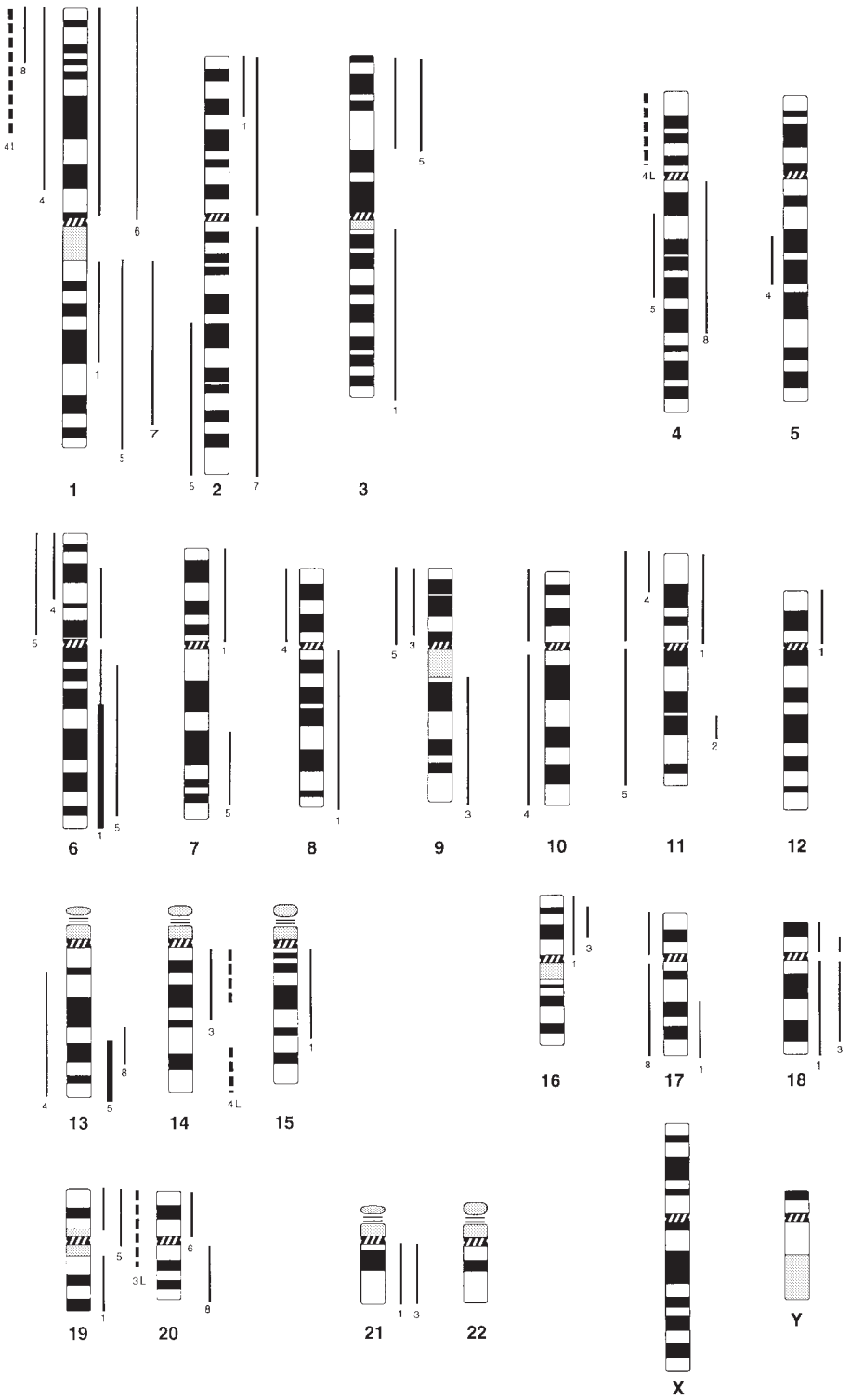

Figure 1. Summary of genetic imbalances detected in 14 leiomyomas and eight leiomyosarcomas. Vertical lines on the left side of each chromosome ideogram represent loss of genetic material in the tumors. Vertical lines on the right side represent gain of genetic material in the tumors. Changes in individual cases are indicated by the case number at the bottom of each line. Solid lines represent leiomyosarcomas, and dotted lines represent leiomyomas. Sites of amplification are represented by heavier lines.

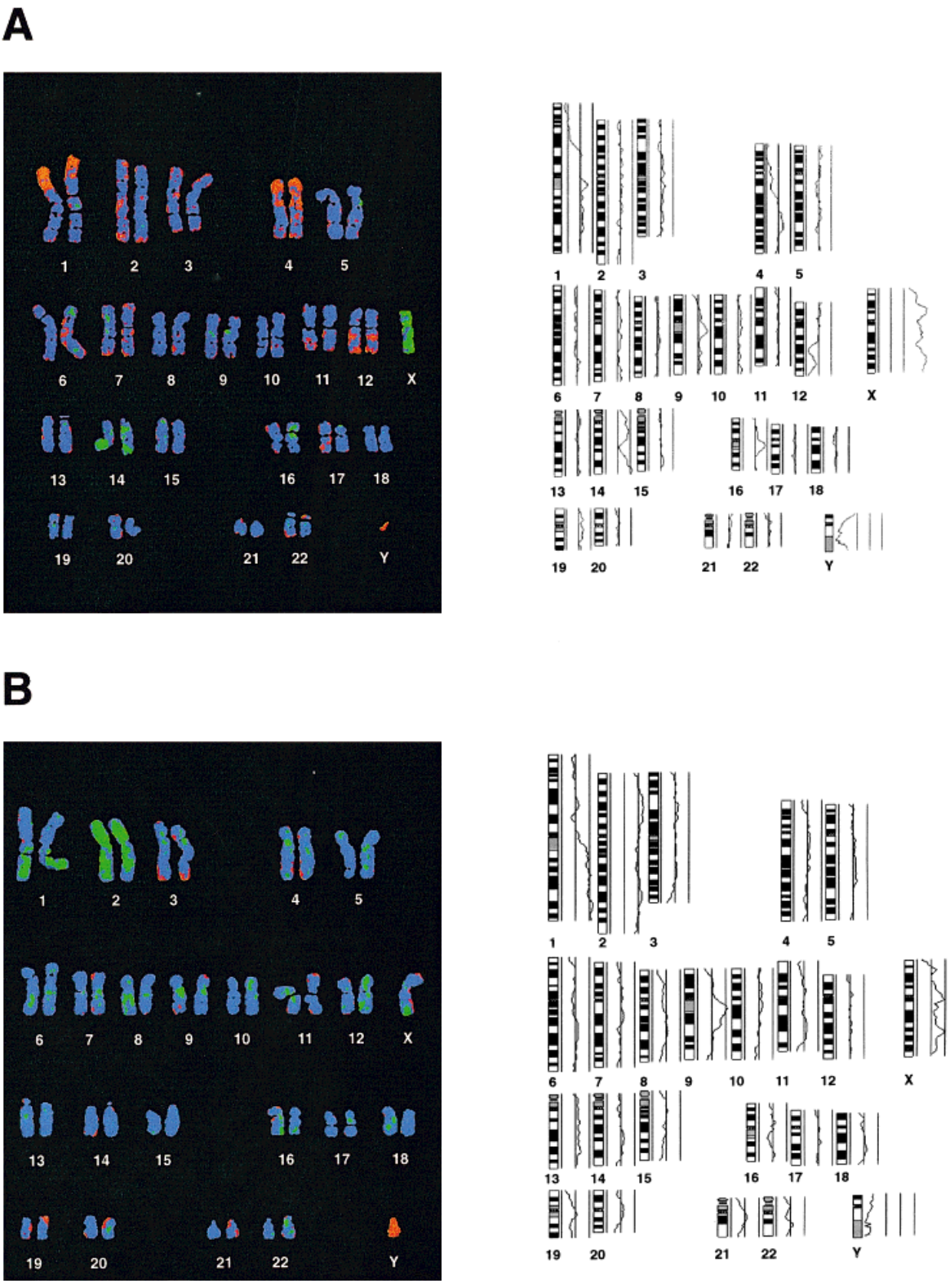

Figure 2. (A) Display of a ratio image and the average ratio profile after CGH of a leiomyoma (case 4L). A multicolor lookup table was used for the visualization of fluorescein/rhodamine ratios: blue indicates a balance between fluorescein and rhodamine values, green indicates an overrepresentation of DNA in the tumor genome, and red indicates a loss of tumor DNA at the corresponding sequences. The chromosomes are displayed in a karyogram-like fashion. In this leiomyoma, there are DNA losses on chromosomes 1p and 4p and DNA gains on chromosome 14q. The average ratio profile was used to identify chromosomal gains and losses. The three vertical lines on the right side of the chromosome ideograms represent fluorescence ratios of 0.75, 1, and 1.25 between the tumor DNA and the normal DNA. The ratio profile curve was computed as a mean of seven metaphase spreads. (B) Display of a ratio image after CGH of a leiomyosarcoma. In this leiomyosarcoma (case 7), there are DNA gains on chromosomes 1q and 2. The average ratio profile was computed as a mean value of eight metaphase spreads.

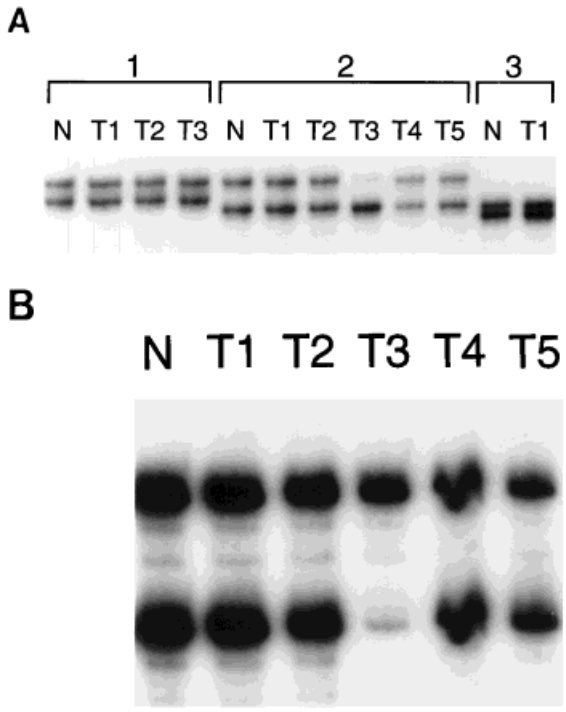

Figure 3. LOH of microsatellite markers on chromosomes 1 and 4 in uterine leiomyomas. Tumor 3 from patient 2 shows LOH at microsatellite markers *D1S1622* (A) and *D4S2366* (B). Tumor 3 is equivalent to sample 4L in the CGH experiment. N, normal DNA; T, tumor DNA.

cytogenetic methodology such as G-band analysis can identify large chromosomal abnormalities such as translocations, deletions, and duplications. However, the tumors must be grown in culture before analysis, and it may be difficult to distinguish between changes that occur in the primary tumor and changes that occur during cell propagation in culture. In the study presented here, we used CGH analysis as a bridge between cytogenetics and molecular genetics. CGH analysis allows the evaluation of cytogenetic changes in primary tumors and gives information on DNA sequence copy number changes, including losses, deletions, gains, and amplifications, although it cannot detect chromosome rearrangements.

Only one of the leiomyomas in this study exhibited a loss on 1p. This result is interesting because cytogenetic studies of both leiomyomas and leiomyosarcomas have detected alterations on this chromosome, and 75% of the uterine leiomyosarcomas in this study showed changes on chromosome 1 [16–24]. The low frequency of 1p alterations detected by CGH and LOH analysis suggests that loss of DNA on chromosome 1p is rare in leiomyomas.

Clinical observations have shown that many women have multiple leiomyomas in their uteri. In two different patients in our study, only one of three leiomyomas exhibited losses or gains of genetic material by CGH. This result supports published data suggesting that leiomyomas arise independently [2].

Another hormonally associated benign tumor of mesenchymal origin is the breast fibroadenoma. Ried et al. [25] analyzed 13 benign breast fibroadenomas by CGH analysis, but none showed changes in DNA copy number. The failure to detect DNA copy number changes in these benign breast tumors indicates that they may not have many changes associated with alterations in DNA copy number. An alternative explanation is that the polyclonal nature of these tumors may prevent identification of genetic alterations because of the small number of cells with the specific aberration. If only a few cells contain the genetic alteration, CGH analysis would not be sensitive enough to detect it [26]. This explanation may also be true for the uterine leiomyomas as well. Also, CGH does not detect copy number loss that is due to mitotic (somatic) recombination.

Because of the rarity of uterine leiomyosarcomas (which make up only 1–3% of all uterine tumors), few of these malignant tumors have been analyzed cytogenetically. Many studies have incorporated cytogenetic data on leiomyosarcomas from various anatomical sites. The most frequent chromosomal alteration observed by G-band analysis and fluorescence in situ hybridization has been structural rearrangement involving chromosome 1. The cumulative data on leiomyosarcomas indicate that these tumors can have very complex karyotypes with much case-to-case variability [27,28]. Additionally, other studies have suggested that the complexity of the karyotype is correlated with the ploidy level of the tumors [19,27]. Although we did not test the ploidy of our tumors, we did notice histologically that leiomyosarcoma sample 3 in our study contained few mitotic figures and had minimal nuclear pleomorphism. CGH analysis of this tumor revealed only one genetic alteration, a gain on chromosome 11q.

All of the uterine leiomyosarcomas in this study showed genetic alterations by CGH analysis. Amplification of 1q21-q22 in this tumor type detected by CGH analysis has been documented by van Kessel et al. [28]. Our study indicated a larger region of amplification on chromosome 1, extending from 1q12 to 1q31 and including additional genetic alterations involving 1p. Both cytogenetic and fluorescence in situ hybridization studies have shown rearrangements and deletion of chromosome 1 in leiomyosarcomas [19–24]. In our study, we did not find any samples in which the entire chromosome was deleted, although there were two samples with partial deletions of 1p. As with the cytogenetic evidence, CGH analysis revealed a complex karyotype with many alterations and much case-to-case variability.

We conclude that the benign uterine leiomyomas have few genetic alterations that are due to DNA copy number changes. In contrast, the leiomyosarcomas appear to have many non-repetitive genetic alter-

ations, suggesting that increased global genetic instability may occur during the progression of this tumor type. However, the specific involvement of chromosome 1 seems to be important in the genesis of this tumor type. By comparing the CGH data from the leiomyomas and leiomyosarcomas, we conclude that there is little genetic evidence of progression from leiomyomas to leiomyosarcomas. Thus, our results support other published data [1,29] that these two tumor types may arise independently. Nonetheless, we cannot exclude the possibility of a common genetic change such as a rearrangement or point mutation in a critical target gene in both tumor types that cannot be detected by CGH. An alteration of this type could result in a dysregulation of growth or cell death in leiomyomas without genetic instability. Thus, the transition of leiomyoma to leiomyosarcoma may be associated with general genetic instability leading to the multiple additional changes seen in the malignant cancers.

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